Interaction of atmospheric ammonia pollution with frost tolerance of plants

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SHORT-TERM EXPOSURE TO ATMOSPHERIC AMMONIA DOES NOT AFFECT LOW-TEMPERATURE HARDENING OF WINTER WHEAT

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Summary

The effect of atmospheric NH_3 on low-temperature hardening of winter wheat (*Triticum aestivum* L. cv. Urban) was investigated. Growth and photosynthesis were stimulated by ammonia exposure. After a 14 day exposure at moderate temperatures (day/night 18.5/16°C) total nitrogen content was enhanced by 45% compared with the controls. During that period, water-soluble sugar content was not affected by NH_3. After lowering the temperature to 4/3°C, sugar content of the control plants doubled within 2 days, whereas in the plants exposed to NH_3 it increased to a much lesser extent. Total nitrogen content further increased, leading to an 85% higher level in the NH_3-exposed plants. Frost hardiness was not affected by atmospheric ammonia. It is concluded that winter wheat is tolerant to high ammonia concentrations, even under unfavourable growth conditions.

Introduction

Air pollution with ammonia, mainly caused by intensive livestock agriculture, is a serious environmental problem in several parts of Europe. It is thought to be one of the reasons for the forest decline (Nihlgård, 1985; Roelofs *et al.*, 1985; Heinsdorf and Krauβ, 1991). In many plant species, large atmospheric concentrations of ammonia might affect growth negatively or lead to a direct injury (Ewert, 1979; Temple *et al.*, 1979; De Temmerman, 1980; Van der Eerden, 1982). On the other hand, usually at smaller NH_3 concentrations, a growth stimulation upon NH_3 exposure was observed (Cowling and Lockyer, 1981; Van der Eerden, 1982; Van der Eerden and Pérez-Soba, 1992; Pérez-Soba and Van der Eerden, 1993).

Frost tolerance of several plant species is affected by air pollution. Exposure of grasses and coniferous trees to SO_2, NO_x, O_3, H_2S or acid mist result in a reduced frost hardiness of the foliar tissue (Baker *et al.*, 1982; Davison and Bailey, 1982; Barnes and Davison, 1988; Mansfield *et al.*, 1988; Fowler *et al.*, 1989; Lucas, 1989; Senser and Payer, 1989; Stuiver *et al.*, 1992; Sheppard *et al.*, 1994; Chappelka and Freer-Smith, 1995). Also, NH_3 exposure might decrease frost tolerance, as was observed in the leaves of some *Brassica* species and the needles of Scots pine trees (Van der Eerden, 1982; Dueck *et al.*, 1991). The negative effect of ammonia on frost tolerance has been attributed to an increased

growth in autumn (Huttunen *et al.*, 1981; Dueck *et al.*, 1991) or a disruption of biochemical and physiological processes (Van der Eerden, 1982; Davison *et al.*, 1988; Dueck *et al.*, 1991). However, the physiological basis for the increased sensitivity to frost after exposure to atmospheric ammonia is largely unclear.

In the present study the response of winter wheat plants to atmospheric ammonia was examined before and during low-temperature hardening, in order to get more insight into the interaction between exposure to ammonia and acclimation to low temperatures. Plants were exposed to 400 and 1000 nl l^{-1} NH_3, concentrations which can occur in The Netherlands during spreading of animal manure (Adema, 1987; Erisman *et al.*, 1987). For the analysis of low-temperature hardening, growth, photosynthesis, total nitrogen and soluble sugar content of the leaves of winter wheat seedlings were measured, since these parameters might influence the hardening process (Levitt, 1980; Gusta *et al.*, 1982; Trunova, 1982).

Materials and methods

Plant material

Seeds of winter wheat (*Triticum aestivum* L., cv. Urban) were sown in commercial potting soil (Florafleur, Nevema, The Netherlands) in plastic pots (diameter 8 cm, volume 0.25 l, five seeds per pot). After 11 days growth in a climate chamber (day/night temperature 20°C/18°C; photoperiod 12 h, photon fluence rate 225 µmol m^{-2} s^{-1} PAR; RH 55%), plants were transferred to cabinets for exposure to NH_3.

NH_3 exposure

Eleven days old plants (11 pots per treatment, five plants per pot) were exposed to 0 (control plants) or 1000 nl l^{-1} NH_3 for 36 days in 150 l stainless steel cabinets with a polycarbonate top as described by Maas *et al.*, (1985). The desired ammonia concentration was achieved by injecting pressurized ammonia (1000 µl l^{-1} in N_2) in the incoming filtered (charcoal filter G7XAD, element 1/1140X, Berko, Assen, The Netherlands) air stream by mass flow controllers (ASM, Bilthoven, The Netherlands). The air exchange rate in the cabinet was 50 l min^{-1}. The air temperature in the cabinet was controlled by circulating cooling fluid through the double cabinet wall and bottom. The air in the cabinet was mixed continuously by a ventilator, in order to reduce the boundary layer resistance. NH_3 concentrations in the cabinets were checked by withdrawal of a known volume of gas from the cabinets and absorption of the NH_3 by bubbling the gas through 0.1 M H_2SO_4. The amount of absorbed NH_3 was determined colorimetrically with the salicylic acid/dichloroisocyanuric acid method according to Willems (1988).

During the first 14 days of the experiment plants were exposed at day/night temperatures of 18.5°C/16°C. Photon fluence rate at plant level was 450 µmol m^{-2} s^{-1} PAR

(Philips HPI-T 400W) for 12 h and relative humidity was 55%. Thereafter, plants were exposed to low temperature and short day conditions (day/night temperature 4°C/3°C; photoperiod 8 h, photon fluence rate 70 µmol m^{-2} s^{-1} PAR; RH 55%) for 22 days.

Plant harvesting

At the beginning of the experiment, before transferring the plants to the NH_3 exposure cabinets, shoots of nine plants were harvested, by cutting the above-ground plant parts at the root-shoot junction. During the next 14 days six shoots per treatment were collected at 2 to 5 day time intervals during exposure to NH_3. Shoots were taken from the pots in such a way that the plant number of all individual pots was kept as high as possible. All harvests were done at the middle of the light period. Immediately after harvesting, total shoot fresh weight was determined, after which the shoots were washed three times with distilled water to remove adsorbed contamination and dried with tissue paper. Subsequently, all the leaves were carefully cut from the shoots and oven-dried for 48 h at 80 °C for determination of soluble sugar and total nitrogen contents.

During the next 22 days, under low-temperature hardening conditions, shoots of three plants per treatment were harvested six times, washed and oven dried (48 h at 80°C) for determination of total nitrogen and soluble sugar contents. Shoot growth was no longer recorded within this period because of the limited number of plants that could be placed in the cabinets and the fact that growth was expected to be low under these conditions (Stuiver *et al.*, 1992; Stuiver *et al.*, 1995). Again, harvests were done at the middle of the light period. Freezing tolerance was determined four times on leaves of three shoots per treatment which were collected 1 hour after the start of the light period.

Determination of total nitrogen and soluble sugar contents

Total nitrogen content was determined on 25 mg oven-dried (48h, 80°C) leaf material from three individual shoots with a modified Kjeldahl method to retain the nitrate during digestion (Doneen, 1932). Samples from each plant were made in duplicate.

Water-soluble sugars were extracted from 25 mg oven dried leaf material of two individual plants per treatment with 80% ethanol, using three extraction steps. The amount of water soluble sugars in the pooled supernatants fraction was determined colorimetrically with anthrone reagent (Fales, 1951). Samples from each plant were made in duplicate.

Photosynthesis measurements

In a separate experiment, photosynthesis was measured on two leaf strips (2*0.7 cm) from the middle of the youngest fully expanded leaf, which had been exposed to 0 and 375 nl l^{-1} NH_3 for 21 days at 18.5/16°C or for 26 days at 4/3°C. The other conditions were the same as in the experiment in which plants were exposed to 0 and 1000 nl l^{-1} NH_3. Photosynthetic oxygen production was measured at 20°C in a temperature- controlled leaf chamber with a Clark-type electrode (Model LD2, Hansatech, Kings Lynn, Norfolk, UK)

under 2% O_2 and 4.5% CO_2 according to Delieu and Walker (1981). Light response curves were made in duplicate for each NH_3 concentration by recording oxygen evolution during 5 min at various photon flux densities up to 1110 µmol m^{-2} s^{-1} PAR.

Determination of freezing tolerance

Freezing tolerance of the leaves was determined after 0, 1, 2 and 3 weeks of hardening at 4/3°C. Eighty leaf strips (1.0*0.7 cm) were placed on moist filter paper (three rows of 16 leaf strips) in an aluminium cuvette (61*12*4 cm), which was cooled to +4°C. Subsequently the temperature was lowered at a rate of 4°C h^{-1}, until a gradient from +4°C until -20°C, with 1.5°C intervals, was established. After reaching a temperature of -20°C, the cuvette was warmed up to 4°C at a rate of 30°C h^{-1} and the leaf strips were thawed for 16 h. Next, leaf strips were placed in Petri dishes on demineralized water (abaxial surface down) in a climate chamber (day/night temperature 20/16°C, 10 h fluorescent light (Osram L 58W/21 and 31; photon fluence rate 100 µmol m^{-2} s^{-1} PAR) to recover.

After 2 days the degree of freezing tolerance of the leaf strips was determined by measuring the photochemical efficiency of photosystem II (F_V/F_M = $(F_M-F_0)/F_M$) with a PAM fluorometer (PAM 101 and 103, H. Walz GmbH, Effeltrich, Germany) after a 30 minutes dark period. F_0 was measured with a modulated light source (PAM 101 ED; peak wavelength 650 nm; light intensity < 1 µmol m^{-2} s^{-1}). F_M was measured at a saturating light intensity of 3000 µmol m^{-2} s^{-1} (light pulse 1 s, Schott KL 1500). The degree of freezing tolerance determined by this method was very similar to the more frequently used electrical conductivity test (R^2 = 0.91). The extent of frost tolerance was assessed as the lowest survival temperature (LST), the lowest temperature where the ratio F_V/F_M was not significantly different from that of the leaves kept at 4 °C.

Statistics

All parameters were statistically analyzed using a two-way analysis of variance (ANOVA). Fresh weight data were analyzed after log-transformation. Table 1 presents the statistical output.

Results

NH_3 exposure at moderate temperatures

The increase in fresh weight was significantly greater in plants exposed to NH_3 than in the clean-air controls (Fig. 1; Table 1). This was reflected in a higher relative growth

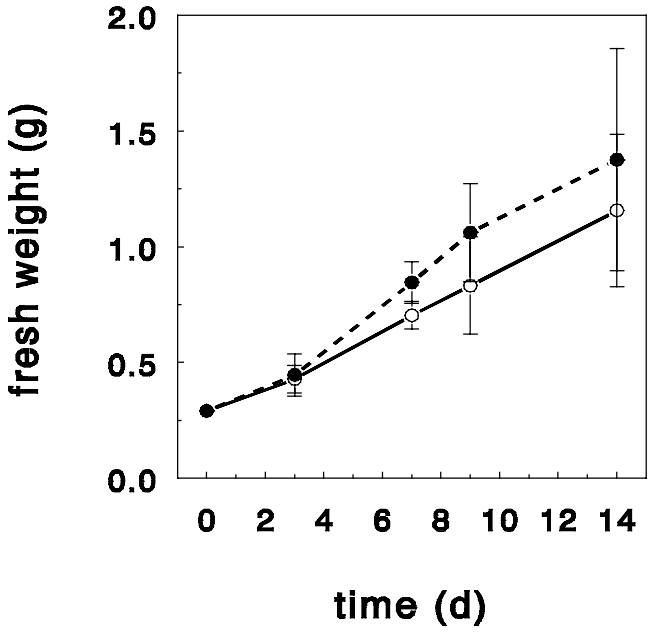

Figure 1. Fresh weight (g) of shoots of winter wheat plants exposed to 0 (○) and 1000 (•) nl l^{-1} NH_3 for 14 days at 18.5/16°C. Means of nine or six replicates ± SD.

*Table 1. Effect of NH_3 exposure on fresh weight, total nitrogen content, soluble sugar content, photosynthesis and freezing tolerance of winter wheat leaves. Results of a two-way ANOVA with exposure time, NH_3 concentration, and their interaction as sources of variance. * $p<0.05$; *** $p<0.001$; n.s.=not significant; n.d.=not determined.*

dependent variable	independent variable		
	time	NH_3	interaction
fresh weight	***	*	n.s.
total N content			
18.5°C	***	***	***
4°C	*	***	n.s.
soluble sugars			
18.5°C	***	n.s.	n.s.
4°C	***	***	***
photosynthesis			
18.5°C	n.d.	***	n.d.
4°C	n.d.	***	n.d.
freezing tolerance	***	n.s.	n.s.

rate, determined over a 14 day time interval, compared with the control plants, namely 0.111 compared with 0.099 g g FW^{-1} d^{-1}.

Exposure to NH_3 resulted in a significantly higher total nitrogen content of the leaves than in control plants (Fig. 2; Table 1). In the control plants the N content of the leaves decreased from day 3 until day 14. Total N content of the NH_3-exposed plants increased until day 7 after which it decreased. As a consequence total nitrogen content of the NH_3 exposed plants after 14 days was 46% higher than the controls (Fig. 2).

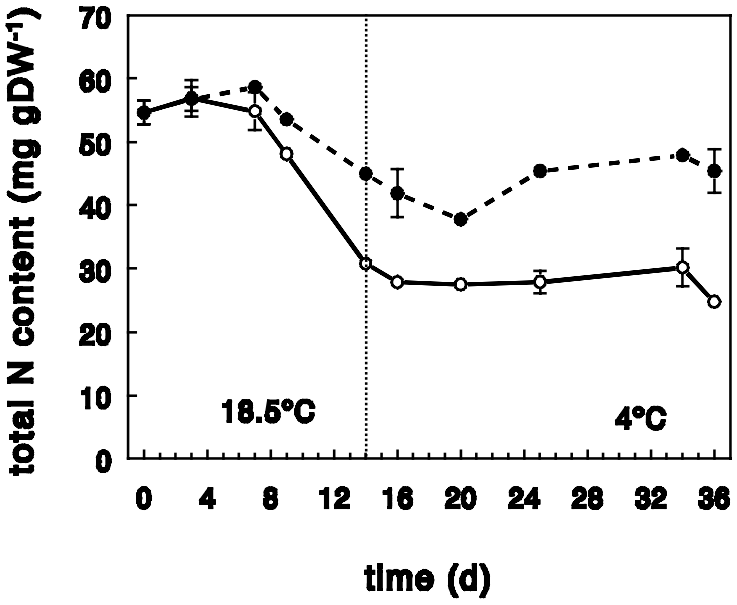

Figure 2. Total nitrogen content (mg g DW^{-1}) of leaves of winter wheat plants exposed to 0 (○) and 1000 (•) nl l^{-1} NH_3 for 14 days at 18.5/16°C and a subsequent 22 days at 4/3°C. Means of three replicates ± SD. If not shown, SD is smaller than symbol.

The water-soluble sugar content almost doubled in both the control and plants exposed to NH_3 during the first 2 weeks of the experiment at 18.5/16°C (Fig. 3). During these 2 weeks at 18.5/16°C, there was no significant difference in sugar content between the treatments (Table 1).

Photosynthesis was higher in leaves of NH_3-exposed plants than the controls at nearly all tested photon flux densities (Fig. 4A). Quantum efficiency was slightly higher in leaves exposed to NH_3 than in the controls, namely 0.076 compared with 0.072 µmol O_2 mol $photons^{-1}$.

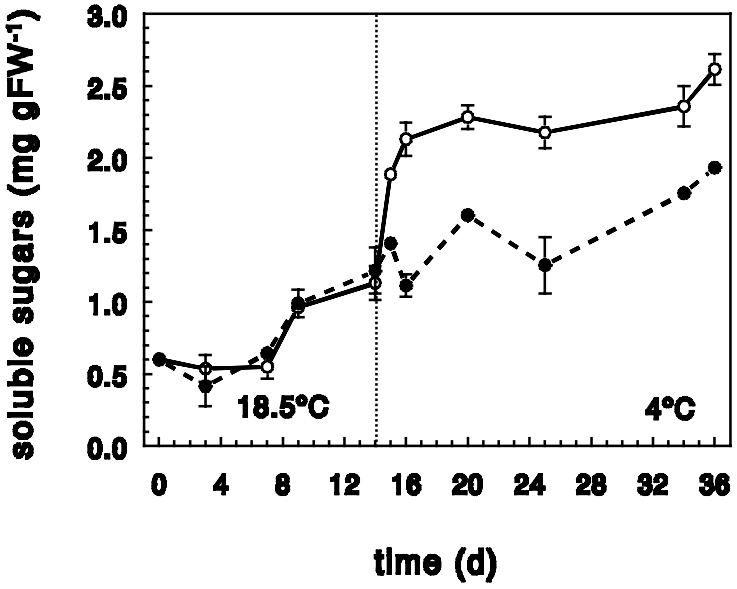

Figure 3. Water-soluble sugar content (mg g FW^{-1}) of leaves of winter wheat plants exposed to 0 (○) and 1000 (•) nl l^{-1} NH_3 for 14 days at 18.5/16°C and a subsequent 22 days at 4/3°C. Means of two replicates ± SD. If not shown, SD is smaller than symbol.

NH_3 exposure at low temperatures

Upon hardening during 3 weeks at 4/3°C, freezing tolerance of the leaves of both the control and the NH_3-exposed plants gradually increased from -3 to -9°C (Table 2). Ammonia exposure did not affect hardening significantly at $p \leq 0.05$ (Table 1).

During exposure to low temperatures, total nitrogen content of the control plants decreased till day 16 after which it remained nearly constant (Fig. 2). In the NH_3-exposed plants a further decrease in total N content was observed after the temperature was lowered, but it was increased again after day 24. This resulted in a 83% higher nitrogen content in the NH_3 plants than in the controls at the end of the experiment (Fig. 2).

After lowering the temperature to 4°C, water-soluble sugar content of the control plants increased rapidly to a content of 2.1 mg g FW^{-1} within 2 days and stayed rather constant at this level in the next 22 days at 4°C. In the NH_3-exposed plants the water-soluble sugar content did not show such a rapid increase during the first 2 days at 4°C. During 11 days of hardening there was a gradual increase but the sugar content remained significantly below the level of 2.6 mg g FW^{-1} of control plants (Fig. 3; Table 1).

Table 2. Frost tolerance (°C), assessed as lowest survival temperature using chlorophyll fluorescence, of winter wheat leaves during hardening for 21 days at 4/3°C. Plants were exposed to 0 and 1000 nl l^{-1} NH_3 for 14 days at 18.5/16°C prior to the hardening and during hardening at 4/3°C. Means of 3 measurements ± SD.

days at 4°C	frost tolerance (°C)	
	0 nl l^{-1}	1000 nl l^{-1}
0	-3.1 ± 0.2	-3.3 ± 0.2
7	-5.0 ± 0.3	-4.6 ± 0.2
14	-6.7 ± 0.3	-7.3 ± 0.3
21	-8.7 ± 0.3	-9.5 ± 0.5

Analogous to the results from moderate temperature-grown plants, in cold-acclimated plants NH_3 exposure lead to a 25% higher photosynthesic rate at the highest photon flux density compared with the controls, namely 27.9 (375 nl l^{-1} NH_3) compared with 22.3 (control) µmol O_2 m^{-2} s^{-1} (Fig. 4B). Cold-acclimated leaves had a higher net O_2-production per unit leaf area than did the leaves from plants grown at 18.5/16°C, which in these plants was respectively 15.9 (control) and 20.6 (375 nl l^{-1} NH_3) µmol O_2 m^{-2} s^{-1} (Fig. 4A and B).

Discussion

At moderate temperatures, exposure to 1000 nl l^{-1} NH_3 resulted in a significantly higher biomass of the plants compared with plants exposed to control air. At the same time total N content of the NH_3 plants was 46% greater than in the controls. Apparently ammonia was taken up by the leaves, as has been demonstrated in leaves of Italian ryegrass (*Lolium multiflorum* Lam.) and poplar (*Populus euramericana* L.) (Lockyer and Whitehead, 1986; Van Hove *et al.*, 1991) and was subsequently metabolized, as evident from the higher biomass. Ammonia can be assimilated to glutamate by the glutamine/glutamate synthase cycle at the expense of carbohydrates (Platt *et al.*, 1977; Champigny *et al.*, 1992; Lea *et al.*, 1992). Stimulation of the enzymes involved in this cycle together with a decrease in carbohydrate content was found in Scots pine (*Pinus sylvestris* L.) trees exposed to ammonia (Pérez-Soba *et al.*, 1994). In winter wheat, at 18.5/16°C, there were no differences in sugar content between the control and NH_3-

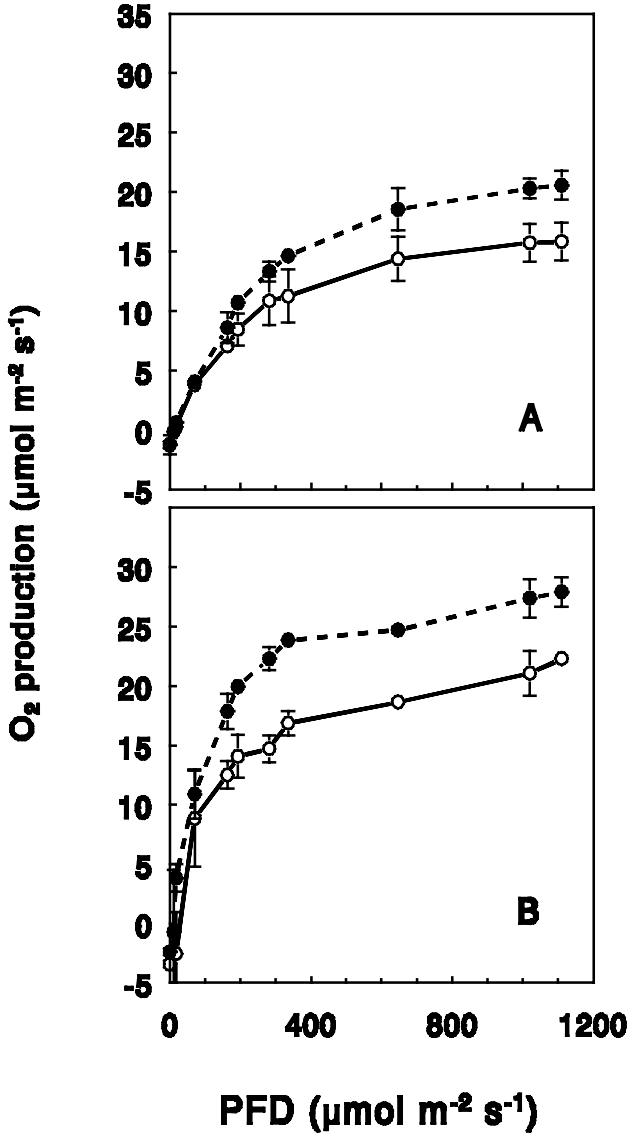

*Figure 4. Photosynthetic rate of two leaf strips (2*0.7 cm) of the youngest fully expanded leaf of winter wheat measured as net O_2-production (µmol m^{-2} s^{-1}) at different photon flux densities, after exposure of plants to 0 (○) and 375 (•) nl l^{-1} NH_3 for 21 days at day/night temperatures of 18.5/16°C (**A**) or 26 days at day/night temperatures of 4/3°C (**B**). For other conditions see 'Materials and methods'. Means of two measurements on two leaf strips ± SD. If not shown, SD is smaller than symbol.*

exposed plants. This might be because in these plants the extra NH_3 assimilation was matched by a higher carbohydrate production resulting from an increased photosynthesis, as was observed in plants exposed to 375 nl l^{-1} NH_3. In this situation metabolization of ammonia possibly might have reduced the sugar content to the level of the control plants.

After decreasing the temperature to 4/3°C the difference in total nitrogen content between control and NH_3-exposed plants increased further. At the same time, ammonia exposure resulted in a much lower water-soluble sugar content than in the control plants during low temperature acclimation, which again indicates that ammonia taken up by the leaves was metabolized (Platt *et al.*, 1977; Champigny *et al.*, 1992; Lea *et al.*, 1992).

In contrast with experiments done with *Brassica* species (Van der Eerden, 1982) or Scots pine trees (Dueck *et al.*, 1991), low-temperature hardening of winter wheat was not significantly decreased by exposure to a gaseous ammonia concentration of 1000 nl l^{-1}. Even with an NH_3 concentration of 2000 nl l^{-1}, no negative effects of ammonia on low temperature hardening of winter wheat could be observed (Clement, unpublished results). Exposure of winter wheat plants to 1000 nl l^{-1} NH_3 did not cause any visible damage to the plants and growth was stimulated by NH_3. In winter wheat the increased growth rate resulting from NH_3 exposure did not lead to a delayed hardening as suggested by several authors (Huttunen *et al.*, 1983; Dueck *et al.*, 1991).

The decrease of water-soluble sugar content of NH_3 exposed winter wheat leaves during hardening at low temperature did not affect freezing tolerance negatively. Also, water-soluble sugar contents of control plants rapidly increased during the first week of hardening, whereas frost tolerance only slightly increased during the first 7 days at 4°C. These results suggest that the accumulation of sugars in winter wheat is not directly related to an increase in frost resistance. As was recently shown by Stuiver *et al.*, (1995), in winter wheat this increase of sugars at low temperatures is probably a result of a disturbed balance between assimilation and growth, which is supported by the observations that processes controlling growth are more sensitive to low temperatures than are processes involved in photosynthesis (Pollock and Eagles, 1988). On the contrary, in *Brassica* there was a good correlation between sugar accumulation and frost hardening (Kohn and Levitt, 1965; Levitt, 1980), which might be the reason for the observed negative effect of NH_3 exposure on frost hardening in this species.

The fact that NH_3 did not affect hardening in winter wheat, whereas it did in Scots pine (Dueck *et al.*, 1991), might possibly be explained by differences in the hardening mechanisms between the two species. In winter wheat low-temperature hardening is induced mainly by a reduction of the growing temperature (Gusta *et al.*, 1982; Trunova, 1982), whereas in Scots pine this process is initiated by a shortening of photoperiod followed by a lowering of the temperature (Bervaes *et al.*, 1978; Christersson, 1978). Possibly, the hardening process during the stage of shortening of the photoperiod is affected more by atmospheric NH_3 than the hardening phase induced by lowering of the temperature. Another explanation might be the fact that plants in our experiments were exposed to NH_3 for only 5 weeks, while in the study of Dueck *et al.* (1991) trees were exposed for 5 or 10 months. During such long exposure periods, other factors might be influenced by NH_3, such as nutrient status of the needles, which might reduce vitality and subsequently the capacity for frost hardening (Nihlgård, 1985; Van Dijk and Roelofs, 1988). Furthermore, it must be realized that winter wheat, as a rapid growing plant, might have a much greater capacity to assimilate internally the absorbed NH_3 than Scots pine trees.

Nevertheless, from the present results we conclude that winter wheat is tolerant to high ammonia concentrations. Neither negative effects on growth and development, nor on low-temperature hardening could be observed during an exposure period of 5 weeks.

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